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14. ABSTRACT Advanced prostate cancer is only temporarily controlled by androgen ablation therapy or chemotherapy, warranting the study of novel approaches. In this regard, recent studies have demonstrated that abnormal growth factor and apoptotic pathways, required by tumor cells to resist multiple insults, can drive tumor cells to even further dependence on glycolysis, supporting a rationale for selectivity of abrogating glycolysis in tumor cells compared to normal cells. Additional recent studies have demonstrated that starvation of tumor cells may induce the process of autophagy, or cell self digestion, and that autophagy may represent a mechanism of tumor cell resistance if allowed to continue only temporarily, followed by cell death if the process of autophagy continues for a prolonged period. In this proposal, we test the hypothesis that modulation of glycolysis will improve clinical results. We therefore hypothesize that 2-deoxyglucose will be safe and active in patients, and abnormal cell pathways such as constitutive activation of Akt, abnormal regulation of autophagy, and other oncogenes may increase sensitivity to inhibition of glycolysis. To test this hypothesis we have the following specific aims: 1. 1. To inhibit glycolysis in patients with prostate cancer in a phase I/II study of 2-deoxyglucose. 2. 2. To determine the mechanism of inhibition of tumor cell growth through modulation of glycolysis.					
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INTRODUCTION:

One in six men will be diagnosed with prostate cancer in their lifetime. For patients that have tumor recurrence following local therapy, all current standard systemic therapies are inadequate due to mechanisms of tumor resistance. In this proposal, we are developing a new paradigm to target metabolism and mechanisms of resistance to agents that induce metabolic stress in prostate cancer. Prior studies demonstrated the dependence of early tumor growth and progression on anaerobic metabolism through glycolysis. In fact, the preference for tumor cells to depend on glycolysis over normal cells is the basis for the successful development of FDG-PET imaging. Despite these prior data, clinical development of agents that target glycolysis has been limited with initial concern over the lack of a therapeutic window. However, more recent studies have demonstrated that abnormal growth factor and apoptotic pathways, required by tumor cells to resist multiple insults, can drive tumor cells to even further dependence on glycolysis, supporting a rationale for selectivity of abrogating glycolysis in tumor cells compared to normal cells. For example, studies have recently demonstrated that activation of Akt kinase, which occurs commonly in tumor such as prostate cancer that are PTEN deficient, increases dependence on glycolysis.

In this proposal, we have been testing the hypothesis that modulation of glycolysis or the induction of metabolic stress will be active against prostate cancer and that the mechanisms responsible for resistance to metabolic stress can be understood. We began to test 2-deoxyglucose, an inhibitor of glycolysis in a phase I clinical trial while conducting parallel studies to understand resistance mechanisms in preclinical models. Our studies from this proposal have demonstrated that starvation of tumor cells may induce the process of autophagy, or cell self digestion, and that autophagy may represent a mechanism of tumor cell resistance if allowed to continue.

Our results have now allowed us to hypothesize that induction of metabolic stress in tumor, as demonstrated with the prototypical glycolytic inhibitor 2-deoxyglucose, will induce autophagy as a resistance mechanism, and future efforts to abrogate autophagy will improve clinical results. We also hypothesize that our initial study of 2-deoxyglucose will be safe and lead to the feasibility of correlative studies in patients, which can be used for future studies of autophagic modulation. Given limited drug supply, we completed the phase I study only, PK, and PD correlative studies. The specific aims of this proposal have been modified to include:

1. To inhibit glycolysis in patients with prostate cancer in a phase I study of 2-deoxyglucose.
2. To determine the mechanism of inhibition of tumor cell growth through modulation of glycolysis.

BODY:

This report represents our final report. New successes from our last annual report in this recent period include full pharmacokinetic data, p62 correlative data and a recently accepted publication of these data (Attached in appendix). Taken together, the full grant period was successful in completing both preclinical and clinical studies and publishing both sets of data in manuscripts. The details of new outcomes in aim 1 and 2 are discussed below:

Aim 1:

In prior reports, clinical trial drug supply and the approval process had delayed trial accrual and the original statement of work was modified and approved to allow more time for the clinical trial approval process and plans to complete the phase I study and correlatives. For imaging correlative studies, we have obtained PET scans prior to study drug and on day 2 to determine if PET is a marker for drug uptake. The hypothesis that was tested here was that 2-deoxyglucose drug should block PET tracer uptake, if the drug is taken up by tumor tissue. This would then be predicted to turn a hot spot on a baseline PET to cold on day 2. In the clinical study, 12 patients have now been treated at doses 30, 45 and 60mg/kg/day orally, enough to complete and publish the phase I study. Therapy was well tolerated with no dose-limiting toxicity. Current toxicity data is shown in Table 2 below, which is from our manuscript accepted for publication in press with an early view (The Prostate, In Press 2010).

TABLE II. Toxicity

Toxicity category	Grade	Number of patients with toxicity		
		30 mg/kg (n = 3)	45 mg/kg (n = 5)	60 mg/kg (n = 4)
Constitutional—fatigue	1/2	1	2	
	3			1
Cardiac—QTc prolongation	1/2	1		1
	3			2
Cardiac—AV block	1/2			1
Cardiac—bradycardia	1/2			1
Gastrointestinal—taste alteration	1/2		1	
Neurological—dizziness	1/2	1	1	1
Pain—gastric pain	1/2			1

Stein et al. The Prostate, 2010 (In Press)

As this was a translational project, laboratory studies informed the clinical trial through the whole project period. Based on our pre-clinical data, we discovered that autophagy was a mechanism of resistance of 2DG and that p62 may be an effective marker for monitoring autophagy. We also demonstrated that approximately 0.5 to 1 mM of 2DG was cytotoxic to prostate cancer cells when autophagy was inhibited by knockdown of Beclin1, an autophagy regulator. Figure 6 from our first manuscript (In Appendix: DiPaola et al. The Prostate 68:1743-1752, 2008), shown below, demonstrates the cytotoxicity of 2DG at various concentrations.

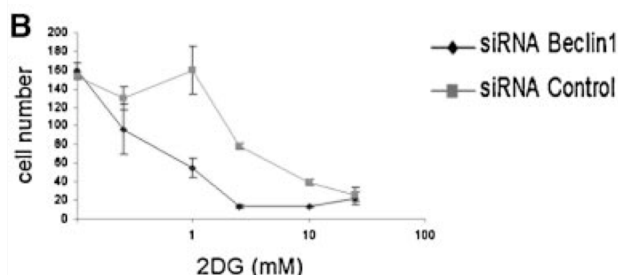


Fig. 6. Effect of autophagy on 2DG induced cytotoxicity. LNCaP (A) and PC-3 (B) cells were treated with various concentrations of 2DG over 72 hr, with and without beclin1 siRNA or Lamin control, and assessed by cell counts with trypan blue. Both LNCaP cells and PC-3 cells were inhibited by 2DG in a dose dependent fashion, and cytotoxicity increased with Beclin1 siRNA. Experiments were performed in triplicate \pm SEM.

To determine the concentrations achievable in man, we developed an assay and assessed 2DG PK in patients. As shown in table 3, below, we were able to achieve 0.6 mM levels at the highest dose concentrations, which support the conclusion that future studies may require autophagic inhibition with an additional autophagy inhibitor along with 2DG. Assessments of PET were also completed as shown in table 4 to establish this as a potential imaging marker for such future studies.

TABLE III. Pharmacokinetics

Dose levels (mg/kg/day)	C _{max} (μ M \pm SD)	AUC (μ mol/L hr \pm SD)	Clearance (ml/hr \pm SD)	t _{1/2} (hr \pm SD)
Cycle 1				
30 (n = 3)	276.7 \pm 18.6	821.2 \pm 236.2	232.0 \pm 63.8	4.5 \pm 0.6
45 (n = 4)	449.2 \pm 168.5	1107.7 \pm 213.3	243.3 \pm 49.4	7.3 \pm 1.6
60 (n = 4)	744.0 \pm 289.1	2086.2 \pm 743.2	185.0 \pm 52.6	5.2 \pm 1.6
Cycle 2				
30 (n = 3)	224.0 \pm 12.6	751.3 \pm 181.7	246.3 \pm 56.6	5.3 \pm 1.1
45 (n = 4)	425.8 \pm 144.9	1087.1 \pm 441.8	270.4 \pm 126.4	8.2 \pm 1.9
60 (n = 4)	605.6 \pm 277.3	1226.9 \pm 571.2	314.9 \pm 108.7	6.1 \pm 3.9

n, number of patients/dose level.

Stein et al. The Prostate, 2010, In Press

TABLE IV. FDG-PET Imaging Marker Assessment

Patient number	Tumor type	Dose level	Tumor site assessed	Baseline SUV (ratio site/liver)	Day 2 SUV (ratio site/liver)
4	Prostate	2	Mediastinum	4.6	4.7
5	Lung	2	Chest	6.3	11.6
6	Cervical	2	Mediastinum	4.3	2
7	Prostate	3	L1 vertebral body	1.2	1
8	Prostate	3	Ischium	2.8	2.0
9	Prostate	3	Mediast	2.1	1.7
10	Prostate	3	Pelvic LN	3.8	4.8
11	Prostate	2	Ilium	3.8	3

Stein et al. The Prostate, 2010, In Press

With an understanding of autophagy on 2DG cytotoxic effects from our preclinical studies, the development of markers of autophagy is now critically important. Based on emerging data in our parallel work (Mathew et al. Cell, 2009), we hypothesized that p62 may be an appropriate autophagic marker for patient studies. Therefore, to determine even the feasibility of measuring p62 in patients for the design of future studies, we assessed p62 in PBMCs of patients as shown in Figure 1 below. Although small in number, these data are important in that they establish feasibility and showed a decrease as would be expected with an agent such as 2DG that induces autophagy.

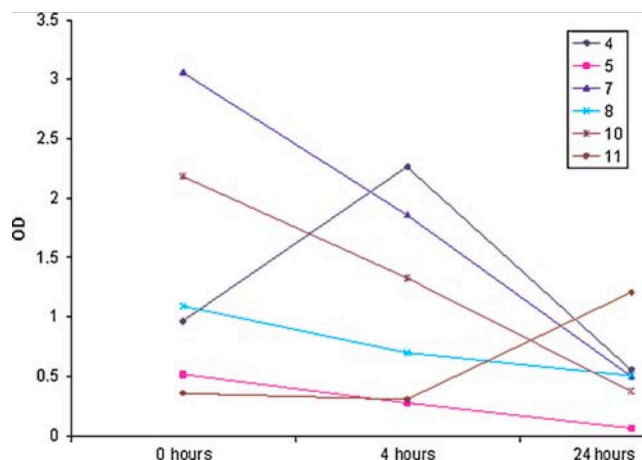


Fig. 1. Assessment of p62 protein levels from peripheral blood mononuclear cells obtained at baseline, 4 hr, and 24 hr in six patients. Optical density of p62 was normalized to α -tubulin.

Stein et al. The Prostate, 2010, In Press

Aim 2:

Within aim 2, we have determined mechanisms of resistance, or activity, of 2-deoxyglucose in prostate cancer. As noted above, we completed a manuscript, which was accepted for publication (Prostate 68: 1743 – 1752, 2008, December 2008). Additional laboratory studies have allowed us to find additional correlative markers such as p62 that could be measured in clinical trials. In fact, we found in the laboratory that in response to stress autophagy-defective tumor cells preferentially accumulate p62/SQSTM1 (p62) protein aggregates, endoplasmic reticulum (ER) chaperones, damaged mitochondria, reactive oxygen species (ROS), and genome damage. Thus, autophagy suppresses oxidative stress and protein, organelle and DNA damage. Suppressing ROS or p62 accumulation provided protection from damage resulting from autophagy defects. Moreover, stress-mediated p62 accumulation caused by defective autophagy stimulated ROS and the DNA damage response and promoted tumorigenesis. These findings suggest that the tumor-suppressive function of autophagy is prevention of sustained p62 accumulation, oxidative damage and genome instability. Thus suppression of autophagy is a mechanism for p62 upregulation. These data are hypothesis generating and allow us to test the hypothesis that p62 will be a marker of the effect of agents that modulate autophagy in clinical trials.

The following represents all completed tasks:

Task 1: Created additional isogenic cell lines (months 1-12). We have completed this task and created these additional isogenic cell lines derived from rat prostate epithelial cells in the laboratory of Dr. E. White (Co-investigator), and created cell lines derived from mouse prostate epithelial cells to take advantage of mutant mice models. Dr. White (Co-PI) published a methods paper on generation of immortal mouse epithelial cell lines including iMPECs (Mathew R, Degenhardt K, Haramaty L, Karp CM, White E. Immortalized mouse epithelial cell models to study the role of apoptosis in cancer. *Methods Enzymol.* 2008;446:77-106).

Task 2: Determined the effect of various oncogenes on glycolysis modulation (Months 7-18). To complete this task, we assessed mechanisms of 2-deoxyglucose effect and discovered that 2-deoxyglucose induces autophagy in prostate tumor cell lines. We also assessed the importance of *beclin1* and *akt* on cytotoxicity and autophagy. This finding is important, given recent studies demonstrating the importance of autophagy as a cell death pathway, or resistance pathway (2). These data are now published in a manuscript (Prostate 68: 1743 – 1752, 2008, December 2008).

Task 3: Determined the effect of various treatments on protein expression (Months 18-36). This was also completed as part of task 2 and is included in the manuscript in the Appendix.

Task 4. Completed the phase I clinical trial to determine phase II dose (Months 24-36). This task is completed with respect to the phase I trial, despite multiple unavoidable delays and limited drug supply, and modified to not include the phase II trial. In fact, these data were already accepted for publication and support future phase II studies. The data supporting autophagy as a resistance mechanism led us to conclude that future studies should be testing a combination of agents that induce metabolic stress such as 2DG along with agents that inhibit

autophagy. Our study was, therefore, helpful to the research community as it determined the feasibility of p62 as a marker for use in these additional combination studies.

Task 5. Determined a phase II dose (Months 30-36). As noted above, our data supports analysis over the next grant period to determine the feasibility of marker assessment for future studies of combination therapy combined with an agent that will abrogate autophagy.

Task 6: Although this task initially planned, to begin a phase II study in patients with hormone refractory prostate cancer (Months 30-36) if drug was available, the limited availability of drug and findings from this project, led us to conclude that a future study should be designed to assess combination therapy, and completion of our part of the initial study phase I study to determine the feasibility of marker assessment in patients.

Task 7: Final Analysis to determine clinical activity of 2-deoxyglucose as a single agent and plan future studies. As noted above, we assessed both activity and marker assessment in the current clinical trial and these data were accepted for publication (In Appendix).

KEY RESEARCH ACCOMPLISHMENTS

1. IND for use of 2-deoxyglucose in clinical trial
2. Full approval of the clinical trial
3. Publication of Jin S, DiPaola RS, White E. Metabolic Catastrophe: A Means to Cancer Cell Death. *J Cell Sci* 2007;120(Pt 3):379-83 (3).
4. Creation of additional isogenic cell lines
5. Finding of a potential mechanism of 2-deoxyglucose cell death modulation
6. Final Preclinical Manuscript Published: Robert S. DiPaola, Dmitri Dvorzhinski, Anu Thalasila, Venkata Garikapaty, Donyell Doram, Michael May, K. Bray, Robin Mathew, Brian Beaudoin, C. Karp, Mark Stein, David J. Foran, and Eileen White. Therapeutic Starvation and Autophagy in Prostate Cancer: A New Paradigm for Targeting Metabolism in Cancer Therapy. *Prostate* 68: 1743 – 1752, 2008, December 2008. (See Appendix).
7. Final Clinical Manuscript accepted for publication with PK and correlative studies (In appendix).

REPORTABLE OUTCOMES:

1. IND for drug use
2. IRB approved Clinical trial
3. Jin S, DiPaola RS, White E. Metabolic Catastrophe: A Means to Cancer Cell Death. *J Cell Sci* 2007;120(Pt 3):379-83
4. Abstract publication ASCO 2007
5. Final Preclinical Manuscript Published: Robert S. DiPaola, Dmitri Dvorzhinski, Anu Thalasila, Venkata Garikapaty, Donyell Doram, Michael May, K. Bray, Robin Mathew, Brian Beaudoin, C. Karp, Mark Stein, David J. Foran, and Eileen White. Therapeutic Starvation and Autophagy in Prostate Cancer: A New Paradigm for Targeting Metabolism in Cancer Therapy. *Prostate* 68: 1743 – 1752, 2008, December 2008. (See Appendix)
6. Clinical Abstract published in ASCO 2008: R. S. DiPaola, M. N. Stein, S. Goodin, S. Eddy, E. H. Rubin, S. Doyle-Lindrud, D. Dvorzhinski, S. Beers, W. J. Shih, E. White Warburg science goes to the bedside: A phase I trial of 2-deoxyglucose in patients with prostate cancer and

advanced malignancies. *J Clin Oncol* 26: 2008 (May 20 suppl; abstr 16087). (See Appendix)

7. Publication acceptance: Targeting Tumor Metabolism With 2-Deoxyglucose in Patients With Castrate-Resistant Prostate Cancer and Advanced Malignancies. Mark Stein, Hongxia Lin, Chandrika Jeyamohan, Dmitri Dvorzhinski, Murugesan Gounder, Kevin Bray, Simantini Eddy, Susan Goodin, Eileen White, and Robert S. DiPaola. *The Prostate*, 2010, In Press.

CONCLUSIONS:

In summary, we completed a productive project that defined the dosing of 2DG in a phase I study, that developed an pharmacokinetic assay and defined the pharmacokinetics of 2DG in patients, demonstrated feasibility of PET as a potential imaging marker for 2DG, completed pre-clinical studies and defined autophagy as a novel mechanism of resistance to 2DG, and already established the feasibility of measuring p62 in patients as a potential marker of autophagy. The discoveries from the laboratory work (identifying autophagy as an important mechanism that may modulate tumor sensitivity to drugs that induce tumor starvation) and clinic (generating an additional hypothesis regarding the importance of PET imaging and p62) have already been significant, and allow for the foundation to design future clinical trials (Stein et al. Manuscript in Press-See attachment in Appendix). We have already been successful in publishing the data from the laboratory portion of this proposal, since the last report, which supports a new paradigm in the treatment of prostate cancer. Based on these data generated in this project, we conclude that future studies should be testing a combination of agents that induce metabolic stress such as 2DG along with agents that inhibit autophagy. In fact, these data from this project, using a prototypical metabolic inhibitor such as 2DG, is hypothesis generating that any agent that induces metabolic stress, such as anti-angiogenesis inhibitors, mTOR inhibitors, or TKIs, should be combined with an agent that inhibits autophagy, supporting a new paradigm that should be tested for the treatment of prostate cancer.

APPENDIX:

Robert S. DiPaola, Dmitri Dvorzhinski, Anu Thalasila, Venkata Garikapaty, Donyell Doram, Michael May, K. Bray, Robin Mathew, Brian Beaudoin, C. Karp, Mark Stein, David J. Foran, and Eileen White. Therapeutic Starvation and Autophagy in Prostate Cancer: A New Paradigm for Targeting Metabolism in Cancer Therapy. *Prostate* 68: 1743 – 1752, 2008, December 2008.

R. S. DiPaola, M. N. Stein, S. Goodin, S. Eddy, E. H. Rubin, S. Doyle-Lindrud, D. Dvorzhinski, S. Beers, W. J. Shih, E. White Warburg science goes to the bedside: A phase I trial of 2-deoxyglucose in patients with prostate cancer and advanced malignancies. *J Clin Oncol* 26: 2008 (May 20 suppl; abstr 16087).

Targeting Tumor Metabolism With 2-Deoxyglucose in Patients With Castrate-Resistant Prostate Cancer and Advanced Malignancies. Mark Stein, Hongxia Lin, Chandrika Jeyamohan, Dmitri Dvorzhinski, Murugesan Gounder, Kevin Bray, Simantini Eddy, Susan Goodin, Eileen White, and Robert S. DiPaola. *The Prostate*, 2010, In Press

Targeting Tumor Metabolism With 2-Deoxyglucose in Patients With Castrate-Resistant Prostate Cancer and Advanced Malignancies

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BACKGROUND. A profound difference between cancer and normal tissues is the preferential utilization of glycolysis by cancer cells. To translate this paradigm in the clinic, we completed a phase I study of 2-deoxyglucose (2DG), and assessed 2DG uptake with fluorodeoxyglucose (FDG) positron emission tomography (PET) and the autophagy substrate p62 as a marker of 2DG resistance.

METHODS. Patients received 2DG orally on days 1–14 of a 21-day cycle in cohorts of three in a dose-escalating manner. Correlative assessments included PET scans at baseline and day 2 and p62 protein in peripheral blood mononuclear cells as a potential marker of 2DG resistance.

RESULTS. The dose of 45 mg/kg was defined as the recommended phase II dose, secondary to dose-limiting toxicity of grade 3 asymptomatic QTc prolongation at a dose of 60 mg/kg. PK evaluation of 2DG revealed linear pharmacokinetics with C_{max} 45 μ g/ml (277 μ M), 73.7 μ g/ml (449 μ M), and 122 μ g/ml (744 μ M) in dose levels 30, 45, and 60 mg/kg, respectively. Five of eight patients assessed with FDG-PET scanning demonstrated decreased FDG uptake by day 2 of therapy, suggesting competition of 2DG with FDG. Five of six patients assessed for p62 had a decrease in p62 at 24 hr.

CONCLUSIONS. These data support the safety of 2DG, defined 2DG PK, demonstrated the effect of 2DG on FDG-PET imaging, and demonstrated the feasibility of assessment of p62 as an autophagic resistance marker. These data support future studies of 2DG alone or in combination with approaches to abrogate autophagy. *Prostate* © 2010 Wiley-Liss, Inc.

KEY WORDS: deoxyglucose; metabolism; prostate cancer; autophagy; p62

INTRODUCTION

The investigation of cancer cell metabolism as a therapeutic target is a rapidly developing research paradigm. The likelihood of therapeutic success by targeting metabolic pathways was predicted in the 1920s when Otto Warburg discovered a major exploitable difference between the metabolism of glucose in normal and cancer cells [1]. Warburg established that cancer cells preferentially utilize the inefficient process of aerobic glycolysis, which can convert each molecule of glucose to two molecules of ATP, rather than oxidative phosphorylation, which can generate up to 36 molecules of ATP. The fact that cancer cells prefer

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glycolysis to convert glucose to ATP forms the basis of tumor imaging with fluorodeoxyglucose (FDG) positron emission tomography (PET) that demonstrates increased glucose uptake in tumor compared to normal tissue. These initial clues to exploitable differences in metabolism highlight the importance of translational drug development focused on targeting metabolic pathways.

Oncogenic events may additionally increase the metabolic fragility of cancer cells. Recent data demonstrate that certain oncogenes exert some of their oncogenic effects through the modulation of the glycolytic pathway, potentially making tumor cells more sensitive to inhibition of glycolysis [2]. These effects have been shown to occur either at the level of glucose uptake through the modulation of glucose transporters or through the direct up-regulation of glycolytic enzymes [3]. Events that promote glycolysis include activation of growth factor receptors, PI-3 kinase, or disruption of PTEN with increased activity of Akt, which also functions as an anti-apoptotic survival factor [4–8]. Growth factors and activated Akt increase surface expression of glucose transporters, stimulate mitochondrial association of hexokinase, and phosphorylate phosphofructokinase, which increase glycolysis [9].

Prostate cancer may be particularly sensitive to modulation of metabolic pathways. Most prostate cancers display altered or deleted activity of PTEN and increased activation of PI3K/Akt signaling [10]. Our prior studies also demonstrated induction of multiple glycolytic enzymes resulting from autocrine stimulation in prostate cancer cells, suggesting that inhibition of glycolysis could exploit the altered metabolism of prostate cancer cells to induce cytotoxicity with an acceptable therapeutic index [11]. Additional studies demonstrated that the glucose analog, 2-deoxyglucose (2DG), an inhibitor of the glycolytic pathway, is cytotoxic to prostate cancer cells in preclinical studies and that the process of autophagy was a significant mechanism of 2DG resistance [12,13].

Taken together, these data support assessment of 2DG in clinical trials and assessment of markers of autophagy as potential clinical markers of drug resistance. Recent studies agree that one mechanism of resistance to therapeutic starvation is the process of autophagy, a response to starvation in which cellular organelles and bulk cytoplasm are targeted to lysosomes for degradation to supply an alternate energy source during periods of nutrient limitation [14]. Our group also demonstrated that the process of autophagy degrades sequestered proteins such as p62, which may serve as a reliable marker of autophagy induction in patients [15].

These prior studies support the translational development of therapeutic starvation in cancer. We

hypothesized that a phase I study with the agent 2DG, as a prototypical glycolytic inhibitor, will provide data on which additional studies can be designed. Furthermore, we hypothesized that we can develop FDG-PET imaging as a marker of drug uptake, define 2DG pharmacokinetics, and establish markers of autophagy as biomarkers of glycolytic inhibitor resistance. We also hypothesized that the development of such markers of autophagy in patients will have high impact for future studies, as research and the importance of autophagy has rapidly grown in recent years [14].

PATIENTS AND METHODS

Patient Eligibility

This study was approved by the local institutional review board. Patients age ≥ 18 years with a histologically proven prostate cancer or other solid tumor malignancy without a standard option of therapy were eligible for this trial. Patients with prostate cancer were maintained on androgen suppression therapy and had disease progression after bicalutamide or flutamide withdrawal of 6 and 4 weeks, respectively. Patients were required to have an ECOG performance status of ≤ 2 and adequate bone marrow, hepatic, and renal function (as defined by granulocytes of $\geq 1,500/\mu\text{L}$, hemoglobin of $\geq 10.0\text{ g/dL}$, platelet count of $\geq 100,000/\text{L}$, normal total bilirubin, aspartate aminotransferase, and alanine aminotransferase of $\leq 2.5 \times$ upper limit of normal, and serum creatinine of $\leq 1.5\text{ mg/dL}$ or creatinine clearance $> 50\text{ mL/min}$). All patients had fasting blood glucose below the institutional upper limit of normal. There was no limit on the number of other prior systemic therapy regimens. Patients with the following conditions were also excluded: patients with a history of glucose intolerance; diabetes or hypoglycemia; seizure disorder; autonomic dysfunction; uncontrolled gastrointestinal disorder; G6PD deficiency; coagulopathies or anticoagulation; second primary malignancies except most in situ carcinomas; known brain metastasis; allergy to methyparaben or propylparaben; active clinically significant infection requiring antibiotics; history of clinically significant unexplained episodes of hypotension, fainting, dizziness, or lightheadedness; history or symptoms of cardiovascular disease (NYHA Class 2, 3, or 4; *New York Heart Association Criteria*) within the last 6 months, particularly coronary artery disease, arrhythmias, or conduction defects with risk of cardiovascular instability, uncontrolled hypertension, clinically significant pericardial effusion, or congestive heart failure; history of transient ischemic attack, stroke, or seizure disorder or any other CNS disease considered to be significant by the investigator; major surgery within 4 weeks of the start of study treatment, without complete recovery; anti-tumor therapy within 28 days of the start

of study treatment; inability to discontinue prohibited medications (including proton pump inhibitors, H₂ antagonists, antacids, drugs for diarrhea or constipation, anti-diabetics; mannitol or sucralfate) for 24 hr before and after dosing on cycle 1, day 1 of weeks 1 and 2; patients who are unable (as per investigator discretion) or unwilling to give written informed consent.

Study Design

This trial was an open-label multiple dose level phase I study. The primary objective was to determine the safety and optimal dose of 2DG administered as a single agent on a daily schedule to patients with advanced solid tumors. Secondary objectives include the pharmacokinetic analysis of 2DG administered on a daily schedule, the effects of 2DG on PET imaging, and the assessment of peripheral blood mononuclear cells (PBMCs) for protein markers of autophagy, as a potential mechanism of resistance of cellular starvation to 2DG.

Dose Escalation and Toxicity Rules

The starting dose for this phase I dose escalation study was 30 mg/kg of 2DG administered orally on a daily schedule for 2 weeks (days 1–14) of a 3-week (21 days) cycle. Dose level 2 was 45 mg/kg. Dose escalation after dose level 2 was determined based on a 33% increase over the previous dose level. Three patients were treated at each dose level until one DLT was observed, at which time the cohort size was planned to be expanded to six patients. If two or more DLTs occur in a cohort of 3–6 subjects, the dose level below is considered the MTD. DLT was defined as any grade ≥ 2 renal; grade 3 or 4 non-hematologic toxicity (including neurotoxicity and asthenia and excluding alopecia, anemia, lymphopenia, or untreated nausea and vomiting) regardless of relationship to 2DG; grade 4 hematologic toxicity for >5 days. DLTs were only considered in the first cycle of therapy (one cycle was 3 weeks) to assess acute toxicity. Patients were assessed for toxicity in other cycles and dose adjusted and reported. Any seizure was considered a DLT. Formal assessment for seizure was at the discretion of the investigator. Hyperglycemia was not considered a DLT unless blood glucose >300 mg/dl for ≥ 6 hr or blood glucose >250 mg/dl for any duration associated with other persistent and significant signs or symptoms such as acidosis. If a persistent (≥ 2 weeks) AE that compromised the patient's ability to participate in the study occurred or if an AE did not resolve to baseline or \leq grade 1, 2DG was discontinued and the patient was discontinued from the study. If >2 dose reductions were needed 2DG was discontinued and the patient was discontinued from the study. Patients experiencing any grade 3 or 4 non-hematologic adverse event

regardless of relationship to 2DG had the dose to be held until the event recovered to baseline or \leq grade 1. If the adverse event did not resolve within 2 weeks, the patient has to be removed from the study. For grade 3 and 4 toxicities, treatment should be withheld until the toxicity resolved to \leq grade 1, then reinstituted (if medically appropriate) at a 25% dose reduction.

Drug Formulation and Administration

2DG was supplied by Threshold Pharmaceuticals in aqueous solution for oral administration. 2DG was stored in a secure area with limited access under controlled conditions including refrigeration (2–8°C). 2DG was supplied in a 40-ml clear glass screw cap vial containing 20 ml nominal (23 ml target fill) of a solution of 2DG formulated at a concentration of 100 mg/ml in water. Each oral dose of 2DG was administered 1 hr before breakfast. Each dose of 2DG was followed by administration of one rinse of the dosing container of approximately 50 ml of water.

Pharmacokinetic, Imaging, and Laboratory Correlative Studies

To determine the ability of imaging to detect 2DG uptake, PET scans were obtained at baseline, C1 day 2, and C2 day 15. To determine 2DG pharmacokinetics, blood samples (5 ml/interval) were collected predose and hourly up to 6 hr following 2DG administration on day 1 and at 24 and 48 hr after day 1 of cycle 1 administration and again on day 1, cycle 2 of the study. For the measurement of 2DG in plasma, the sample extracts were derivatized to 2-aminobenzoic acid derivatives, and determined using highly sensitive high-performance liquid chromatography fluorometric method (HPLC-FL). To assess protein markers of autophagy, PBMCs were collected and assessed for protein expression by Western blot. Assessment for p62 was done to assess the feasibility of obtaining this marker of autophagy, as previously demonstrated in our prior studies [15,16].

For assessment of p62, PBMC cell extracts were made in Laemmli buffer and separated by SDS-PAGE (30 μ g protein) and transferred to polyvinylidene fluoride (PDVF) membranes (Millipore, Billerica, MA). Blots were incubated with primary antibodies p62 (Biomol, Plymouth Meeting, PA), α -tubulin (Sigma, St. Louis, MO) and developed with HRP-conjugated secondary antibody using the ECL system (GE Healthcare, Piscataway, NJ). The protein expression images were scanned on HP Scanjet 4570c scanner with the following optical density analyses using ImageJ software (NIH, Bethesda, MA). Results for p62 were normalized by α -tubulin.

TABLE I. Patient Characteristics

Patient characteristics	No. of patients
Total	12
Male	11
Female	1
Age (years)	
Median	65
Range	36–83
ECOG performance status	
0	8
1	4
Primary site of disease	
Prostate	9
Nasopharynx	1
Lung	1
Cervix	1
Prior chemotherapy regimens	
0	6
1	1
2	3
3	1
Greater or equal to 4	1

RESULTS

Patient's Characteristics

Twelve patients (Table I) received treatment of 2DG within one of three dose levels (30, 45, and 60 mg/kg). The median age of patients was 65.5 years. Nine patients were diagnosed with advanced castrate-resistant prostate cancer (CRPC), one with cervical cancer, one with nasopharyngeal cancer, and one with non-small cell lung cancer. Six patients had received and progressed after prior chemotherapy. Of the nine patients with prostate cancer, all had progression on prior androgen ablation therapy and three had progression after at least one prior chemotherapy regimen.

Adverse Events and Clinical Outcomes

Therapy was well tolerated with few grade 3 or 4 toxicities, as shown in Table II. The most common grade 1 and 2 toxicities were fatigue and dizziness (grade 2 fatigue in three patients and grade 1 dizziness in two patients). Dose-limiting toxicity of grade 3 asymptomatic QTc prolongation was seen in two patients treated at a dose of 60 mg/kg. Therefore, two additional patients were safely enrolled at the dose of 45 mg/kg. None of the five patients dosed at 45 mg/kg had a DLT and 45 mg/kg was selected as the recommended phase II dose. In seven patients with prostate cancer who have completed study therapy, no declines in PSA were seen. Three patients with CRPC remained on study beyond three cycles with radiographically stable disease (8, 12, and 21 cycles).

Pharmacokinetics

Pharmacokinetics of 2DG for cycles 1 and 2 are shown in Table III. Mean AUC in the first cycle was 821.2 ± 236.2 , 1107.7 ± 213.3 , and 2086.2 ± 743.2 ($\mu\text{mol/L hr} \pm \text{SD}$) at doses of 30, 45, and 60 mg/kg, respectively. The C_{max} in the first cycle also increased with increasing dose levels, which were 276.7 ± 18.6 , 449.2 ± 168.5 , and 744.0 ± 289.1 ($\mu\text{M} \pm \text{SD}$) in dose levels 30, 45, and 60 mg/kg, respectively. The half-life at the recommended phase II dose level of 45 mg/kg was 7.3 ± 1.6 in cycle 1 and 8.2 ± 1.9 (hr $\pm \text{SD}$) in cycle 2.

Imaging and Molecular Markers

Nine patients underwent assessment with FDG-PET scanning before treatment and on day 2 to determine the effect of 2DG on FDG uptake (Table IV). Using the ratio of SUV at selected tumor sites over the liver, as a control, decreased FDG uptake occurred in five patients in either dose level 2 (patients numbered 6 and 11) or 3 (patients numbered 7, 8, and 9). Based on prior studies demonstrating the effect of autophagy on

TABLE II. Toxicity

Toxicity category	Grade	Number of patients with toxicity		
		30 mg/kg (n = 3)	45 mg/kg (n = 5)	60 mg/kg (n = 4)
Constitutional—fatigue	1/2	1	2	
	3			1
Cardiac—QTc prolongation	1/2	1		1
	3			2
Cardiac—AV block	1/2			1
Cardiac—bradycardia	1/2			1
Gastrointestinal—taste alteration	1/2		1	
Neurological—dizziness	1/2	1	1	1
Pain—gastric pain	1/2			1

TABLE III. Pharmacokinetics

Dose levels (mg/kg/day)	C _{max} (μM ± SD)	AUC (μmol/L hr ± SD)	Clearance (ml/hr ± SD)	t _{1/2} (hr ± SD)
Cycle 1				
30 (n = 3)	276.7 ± 18.6	821.2 ± 236.2	232.0 ± 63.8	4.5 ± 0.6
45 (n = 4)	449.2 ± 168.5	1107.7 ± 213.3	243.3 ± 49.4	7.3 ± 1.6
60 (n = 4)	744.0 ± 289.1	2086.2 ± 743.2	185.0 ± 52.6	5.2 ± 1.6
Cycle 2				
30 (n = 3)	224.0 ± 12.6	751.3 ± 181.7	246.3 ± 56.6	5.3 ± 1.1
45 (n = 4)	425.8 ± 144.9	1087.1 ± 441.8	270.4 ± 126.4	8.2 ± 1.9
60 (n = 4)	605.6 ± 277.3	1226.9 ± 571.2	314.9 ± 108.7	6.1 ± 3.9

n, number of patients/dose level.

p62 protein levels, assessment of p62 was assessed in PBMCs of patients before and during treatment with 2DG. Of six patients with PBMCs available for assessment of p62 by immunoblot, five of six patients had a decrease in p62 level at 24 hr (Fig. 1).

DISCUSSION

This is the first study to define the recommended phase II dose, pharmacokinetics, and molecular marker data for 2DG, a prototypical inhibitor of glycolysis, as a single agent. The ability to use 2DG in the clinic lays an important foundation in the effort to target metabolism in cancer. While the abnormal metabolism of cancer cells has long been recognized, the underlying genetic abnormalities driving aberrant metabolism are starting to come into focus necessitating the development of clinical agents that can exploit metabolic differences between cancer cells and the normal host environment. 2DG has long been recognized as an effective inhibitor of glucose metabolism. When 2DG is taken into the cell, 2DG is phosphorylated to 2DG-P, which is trapped in the cell and cannot be utilized for energy, leading to inhibition of glycolysis and depletion of cellular ATP [17–21].

Multiple preclinical studies have evaluated the efficacy of 2DG as a single agent and have identified

agents that may effectively be combined with 2DG to enhance efficacy. In vitro studies with 2DG have demonstrated activity in osteosarcoma cells that were defective in oxidative phosphorylation implying that cells relying on glycolysis are more susceptible to 2DG [12]. Other studies have demonstrated that 2DG increased the efficacy of death receptor mediated apoptosis with TNF and TRAIL [22]. In vivo, 2DG has enhanced the cytotoxicity of adriamycin and paclitaxel in osteosarcoma and lung cancer xenograft models [23], and increased cytotoxicity when combined with the microtubule inhibitor 2-methoxyestradiol [24]. 2DG has also been combined with radiation preclinically and in clinical trials where 2DG was administered prior to treatment with high-dose radiation fractions administered on a weekly basis [25]. More recently, our group demonstrated the cytotoxic effect of 2DG in prostate cancer cell lines and demonstrated that the process of autophagy was a significant mechanism of resistance to 2DG [16].

In our clinical study, 2DG was well tolerated at the recommended phase II dose of 45 mg/kg. At the higher dose of 60 mg/kg, the QT interval was prolonged. This finding is in agreement with prior data demonstrating prolonged QT interval in studies of 2DG stimulation of gastric acid production. In this prior study, when used

TABLE IV. FDG-PET Imaging Marker Assessment

Patient number	Tumor type	Dose level	Tumor site assessed	Baseline SUV (ratio site/liver)	Day 2 SUV (ratio site/liver)
4	Prostate	2	Mediastinum	4.6	4.7
5	Lung	2	Chest	6.3	11.6
6	Cervical	2	Mediastinum	4.3	2
7	Prostate	3	L1 vertebral body	1.2	1
8	Prostate	3	Ischium	2.8	2.0
9	Prostate	3	Mediast	2.1	1.7
10	Prostate	3	Pelvic LN	3.8	4.8
11	Prostate	2	Ilium	3.8	3

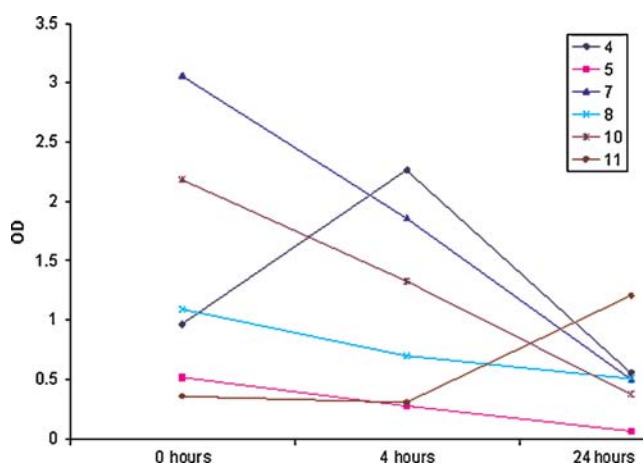


Fig. 1. Assessment of p62 protein levels from peripheral blood mononuclear cells obtained at baseline, 4 hr, and 24 hr in six patients. Optical density of p62 was normalized to α -tubulin.

as a vagal stimulator to test for completeness of surgical vagotomies, 2DG induced non-specific T wave flattening and QT prolongation, without inducing serious arrhythmias [26]. In our study, we utilized a schedule of daily dosing for 2 weeks with 1 week off to allow recovery from induction of glucopenia. The PK was linear with dose level, demonstrated a C_{max} median of 744 μ M at the highest dose level, and had relatively little inter-patient variability. Although this study supports the safety of 2DG as a single agent at blood concentrations that reach 0.7 mM concentrations, our prior laboratory studies demonstrated that cytotoxic effects below 1 mM were enhanced by the abrogation of autophagy as a mechanism of resistance of 2DG [16]. These data, therefore, suggest that future studies of 2DG may be best approached in combination with an autophagy inhibitor.

In fact, the process of autophagy is now known to be a cell survival mechanism to multiple agents capable of tumor cell starvation including 2DG, mTOR inhibitors, VEGF, and tyrosine kinase inhibitors [14]. More recently, our group demonstrated that autophagy regulates the expression of p62, suggesting that this may be a potential surrogate clinical marker of autophagy [15]. In an effort to begin to test this hypothesis that autophagy decreases the expression of p62, we assessed the feasibility of measuring p62 in patients PBMCs. As shown in Figure 1, p62 decreased in 5/6 patients assessed by 24 hr. Although only a small cohort of patients, these data are important and form the basis to test p62 as a marker of the modulation of autophagy in larger studies. In our study, FDG-PET scans also demonstrated possible competitive inhibition of glucose uptake in the majority of patients, which may be tested further as a useful pharmacodynamic marker.

In summary, this phase I and correlative study defined the recommended phase II dose of 2DG, defined 2DG pharmacokinetics, assessed the effect of 2DG on FDG-PET, and determined the feasibility of assessment of p62 as a potential marker of autophagy. Given parallel emerging laboratory data on autophagy as a mechanism of resistance to many agents capable of tumor cell starvation, the feasibility of measuring p62 as a marker of autophagy is additionally important for the development of future studies. Future studies with 2DG in combination with agents that abrogate autophagy are also warranted.

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Therapeutic Starvation and Autophagy in Prostate Cancer: A New Paradigm for Targeting Metabolism in Cancer Therapy

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BACKGROUND. Autophagy is a starvation induced cellular process of self-digestion that allows cells to degrade cytoplasmic contents. The understanding of autophagy, as either a mechanism of resistance to therapies that induce metabolic stress, or as a means to cell death, is rapidly expanding and supportive of a new paradigm of therapeutic starvation.

METHODS. To determine the effect of therapeutic starvation in prostate cancer, we studied the effect of the prototypical inhibitor of metabolism, 2-deoxy-D-glucose (2DG), in multiple cellular models including a transfected pEGFP-LC3 autophagy reporter construct in PC-3 and LNCaP cells.

RESULTS. We found that 2DG induced cytotoxicity in PC-3 and LNCaP cells in a dose dependent fashion. We also found that 2DG modulated checkpoint proteins cdk4, and cdk6. Using the transfected pEGFP-LC3 autophagy reporter construct, we found that 2DG induced LC3 membrane translocation, characteristic of autophagy. Furthermore, knockdown of beclin1, an essential regulator of autophagy, abrogated 2DG induced autophagy. Using Western analysis for LC3 protein, we also found increased LC3-II expression in 2DG treated cells, again consistent with autophagy. In an effort to develop markers that may be predictive of autophagy, for assessment in clinical trials, we stained human prostate tumors for Beclin1 by immunohistochemistry (IHC). Additionally, we used a digitized imaging algorithm to quantify Beclin1 staining assessment.

CONCLUSIONS. These data demonstrate the induction of autophagy in prostate cancer by therapeutic starvation with 2DG, and support the feasibility of assessment of markers predictive of autophagy such as Beclin1 that can be utilized in clinical trials. *Prostate* 68: 1743–1752, 2008. © 2008 Wiley-Liss, Inc.

KEY WORDS: prostate cancer; deoxyglucose; beclin1; autophagy; glycolysis; metabolism

INTRODUCTION

The relentless progression of advanced prostate cancer is only temporarily disrupted by current therapeutic interventions including androgen ablation therapy or chemotherapy [1,2]. Prostate cancer cells become resistant by inactivating normal pathways of cell death and activating pathways of cell survival, but our knowledge of these mechanisms is incomplete.

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One area that has received renewed attention is the metabolic fragility of cancer cells, which preferentially utilize glycolysis to metabolize glucose rather than oxidative phosphorylation. This difference, initially termed "The Warburg effect," remains one of the fundamental features that distinguishes normal cells from tumor cells [3,4]. Whereas aerobic metabolism can generate 36 molecules of ATP per molecule of glucose, anaerobic glycolysis can only generate two. This fragility is magnified because cancer cells must survive in hostile environments with poor blood supply, limited oxygen, reduced growth factors, limited nutrients, and high metabolic demand. Our prior studies demonstrated induction of multiple glycolytic enzymes resulting from autocrine stimulation specifically in prostate cancer cells, suggesting that inhibition of glycolysis would exploit the metabolic fragility of prostate cancer [5].

Few studies to date have been completed with agents that directly target glycolysis to induce cytotoxicity, despite diagnostic studies developing positron emission tomography (PET), which uses a trapped glucose analogue, 2-deoxy-D-glucose (2DG), for detection of tumor. For example, Liu et al. [6] demonstrated that osteosarcoma cells that were defective in oxidative phosphorylation were 10-fold more sensitive to 2DG and 5-fold more sensitive to oxamate compared to wild type cells, demonstrating that cells relying on glycolytic pathways are sensitive to these anti-glycolytic agents. Munoz-Pinedo et al. [7] demonstrated that 2DG enhanced apoptosis induced by tumor necrosis factor, CD95 agonistic antibody and TRAIL in multiple tumor cell lines. Aft et al. [8] demonstrated activity of 2DG in an in vivo mouse breast tumor model. Additionally, Kurtoglu et al. [9] supported the hypothesis that additional mechanisms of cytotoxicity of 2DG occur including inhibition of N-linked glycosylation. As further studies of agents that inhibit glycolysis, such as 2DG, are ongoing in the laboratory and the clinic, an understanding of additional potential mechanisms of activity and drug resistance will be important.

In this regard, the process of autophagy, which is induced by nutrient deprivation, has been identified as an important mechanism of cellular resistance, or alternatively cell death if allowed to continue unabated [10–12]. Autophagy is a response to starvation whereby cellular organelles and bulk cytoplasm are targeted to lysosomes for degradation to supply an alternate energy source during periods of nutrient limitation. In addition to nutrient recycling, autophagy also plays an essential role in the proteolytic degradation of damaged proteins and organelles to maintain quality control. Sustained autophagy under conditions of protracted starvation has also been proposed to lead to cell death; thus, the survival or death consequences

of autophagy are condition-dependent. Autophagy is also often impaired in human prostate cancers, due to either activation of the PI-3 kinase/Akt pathway and thereby mTOR, which inhibits autophagy, or through allelic loss of the essential autophagy gene *beclin1* [11]. Therefore, growth in a hostile environment, inefficient utilization of glucose and defective autophagy predict that prostate cancers may be particularly sensitive to therapies that inflict metabolic stress.

We, therefore, hypothesize that prostate cancer is metabolically fragile because of dependence on glycolysis, increased activity of Akt, and impaired autophagy. This creates an opportunity to improve therapy through promotion of metabolic stress with agents that inhibit glycolysis. To begin to understand this novel paradigm, we studied the effect of a prototypical inhibitor of glycolysis, 2DG, a glucose analogue that inhibits glucose uptake, to determine if, in fact, 2DG induces cytotoxicity and autophagy in prostate cancer cells. To develop markers of autophagy for assessment in clinical trials, we studied Beclin1 in our cell systems and human prostate tissue from patients with prostate cancer.

MATERIAL AND METHODS

Cell Culture and Viability Assay

PC-3 (human androgen insensitive prostate cancer cell line), LNCaP (human androgen sensitive prostate cancer cell line) were obtained from ATCC. LNCaP and PC-3 cells were maintained in RPMI-1640 media with glucose concentration 2 g/L and 10% FBS. 2DG was obtained from Sigma (St. Louis, MO). Cells were plated initially in 96-well microtiter plates. After 24 hr they were treated with different concentrations of 2DG. After 72 hr of incubation in the presence or absence of drug, viability studies were performed by the MTT method as previously described [13]. Trypan Blue exclusion viability assay was performed on cells plated in 100 mm dishes. Cells were removed with trypsin after 72 hr and triplicate samples from each dish were counted on VI-Cell (Beckman Coulter Fullerton, CA). Mean and standard error was calculated. Time lapse microscopy was performed as previously described [14].

RNA Interference

LNCaP and PC-3 cells were transfected with annealed, purified, and desalted double-stranded siRNA (30 µg/3 × 10⁶ cells) using the Amaxa nucleofection system (Gaithersburg, MD) (kit V, program G-16), as previously demonstrated [14]. siRNA targeted against *beclin1* (5'-CAGUUUGGCACAAUCAUAUU-3') and

LaminA/C were obtained from Dharmacon Research (Lafayette, CO).

Fluorescence Microscopy/LC3-GFP Autophagy Assay

LNCaP and PC-3 cells were co-transfected with EGFP-LC3 reporter along with *LaminA/C* siRNA (control) or *beclin1* siRNA and plated on cover slips, treated with 2DG and cultured in maintenance media. After 72 hr cover slips were fixed in Formalde-Fresh solution (Fisher Scientific, Pittsburgh, PA). Following the washing and mounting the cover slips, the cells with GFP translocation were counted (>30 total) and photographed using fluorescence microscope (Nikon).

Immunoblot Analysis

Cells treated with 2DG were lysed in ice-cold RIPA buffer with protease inhibitors cocktail from (Sigma). Equivalent amounts of protein from each sample were electrophoresed on 12% or 15% gel SDS-PAGE and transferred to nitrocellulose. For cell cycle protein assessment, Cyclin D1, Cdk4, Cdk6 and secondary goat anti-mouse HRP conjugated antibody were used (Sigma). Beclin1 was assessed using rabbit primary antibody (Santa Cruz Biotechnology, Inc., cat # sc-11427, Santa Cruz, CA). LC3 was assessed using primary rabbit antibody from MBL International (Woburn, MA) and secondary goat anti-rabbit HRP conjugated antibody (Santa Cruz Biotechnology, Inc.). Cleaved Caspase3 antibody was obtained from Cell Signaling (Beverly, MA).

Immunohistochemistry of TMA

Tissue microarray slides with paraffin embedded prostate cores were placed into a Ventana Medical Systems Discovery automated slide stainer, heated to 75°C for 8 min. Deparaffinization (de-waxing) of tissues is accomplished using heat and Ventana de-waxing solutions for 8–10 min. Slides were washed in buffer at 37°C for 10 min. Antigen retrieval is performed for over 72 min at a pH 8 using EDTA buffer. Anti-BECN1 (H-300) (Santa Cruz Biotechnology, Inc., cat # sc-11427) was applied to the tissue sections at a dilution of 1:240 with 1% BSA/PBS with amplification and incubated overnight at room temperature. Biotinylated secondary antibody (Discovery Universal detection #2) was applied to the tissue sections and developed with Ventana Strept-Avidin Horseradish Peroxidase. Hematoxylin was used as a tissue counterstain.

Imaging

Immunostained tissue microarrays were imaged and digitized using a 40× volume scan on a high-

throughput Trestle/Clariant MedMicro whole slide scanner. The resulting imaged specimens were stored in multi-tiled TIFF format on a redundant array of independent devices (RAID). Tissue Microarray analysis software automatically performs registration of the arrays, decomposes the specimen into its constituent staining maps and generates the measures for integrated staining intensity (ISA), effective staining area (ESA), and effective staining intensity (ESI) [15]. The software manages imaged tissue microarrays along with all related descriptive text and data fields into an Oracle 10 g database. The expression metrics that are generated during processing are automatically populated into the database and can be used to query and locate any given imaged specimen and correlated dataset to facilitate subsequent retrieval.

RESULTS

Effect of 2DG in Cancer Cell Lines

To determine if 2DG inhibits prostate cancer cell viability, we treated LNCaP and PC-3 cells with increasing concentrations of 2DG. As shown in Figure 1, both LNCaP cells and PC-3 cells are inhibited by 2DG in a dose dependent fashion. The cytotoxic effect was demonstrated by cell counts using a trypan blue assay (Fig. 1A) and an MTT cell viability assay (Fig. 1B). To determine the effect of 2DG over time, we observed morphological changes by time-lapse microscopy over 5 days (100×). As shown in Figure 1C, proliferation was decreased more by therapeutic starvation with exposure of cells to 2DG (bottom two rows) compared to control (top row) or compared to cells in which glucose was absent from the media (although present in added serum). To determine the effect of 2DG on cell cycle checkpoint proteins, we assessed the effect of 2DG on expression of Cyclin-D1, cdk4, and cdk6. As shown in Figure 2, 2DG decreased expression of Cyclin-D1, cdk4, and cdk6 in both LNCaP and PC-3 cells. Thus, 2DG arrests cell growth and promotes cell death of prostate cancer cell lines in a dose-dependent fashion.

Autophagy and Beclin-1 in Tumor Cell Lines

To determine the effect of 2DG on autophagy, we expressed the fluorescent autophagy marker GFP-LC3 in LNCaP and PC3 cells, and modulated the expression of the autophagy regulator Beclin1 with siRNA. As shown in Figure 3A, siRNA for Beclin1 efficiently decreased expression in PC-3 cells compared to control siRNA. Treatment of 2DG induced autophagy as demonstrated by redistribution of the autophagosome marker GFP-LC3 from a diffuse cytoplasmic pattern to

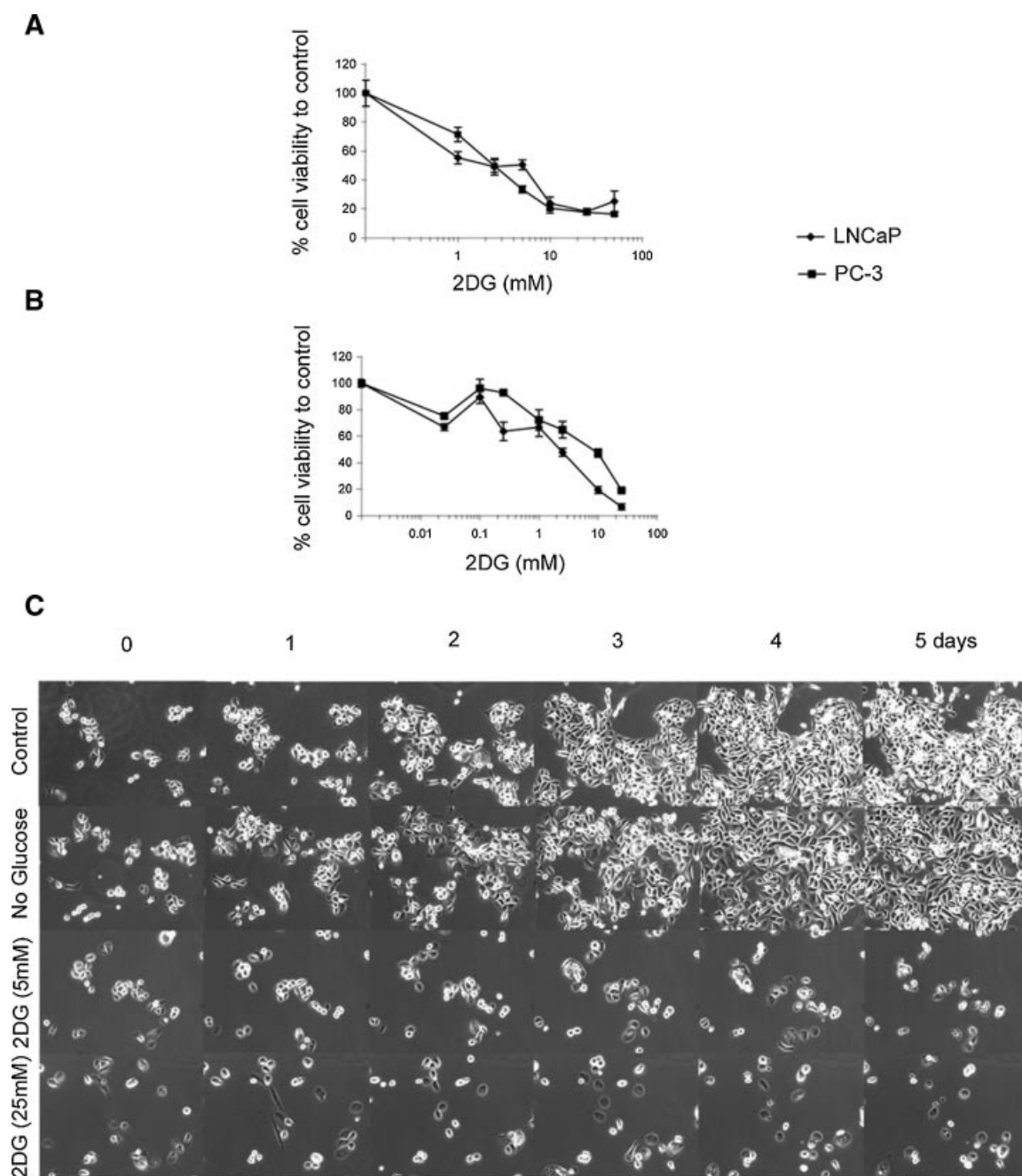


Fig. 1. Effect of 2DG on prostate cancer cell viability. LNCaP and PC-3 cells were treated with various concentrations of 2DG over 72 hr and assessed by cell counts with trypan blue (**A**) and MTT assay (**B**). Both LNCaP cells and PC-3 cells were inhibited by 2DG in a dose dependent fashion. Experiments were performed in triplicate \pm SEM. To determine the effect of 2DG on cellular morphology, morphological changes in PC-3 cells were observed with time-lapse microscopy (100 \times) (**C**). Cells were treated with vehicle (Control), no glucose in media (except that contained in added serum), 5 and 25 mM 2DG over 5 days.

form punctate localization indicative of autophagosome formation (Fig. 3B shows a photograph of representative cells and C quantification of percentage of cells with punctate redistribution). In Figure 3B, the arrows identify punctate localization in cells treated with 2-DG, which do not develop with Beclin1 siRNA treatment (second row of Fig. 3B). As shown in

Figure 3C, over 40% of cells contain such punctate GFP-LC3 localization with 5 mM 2DG, which is decreased to less than 20% of cells with the addition of Beclin1 siRNA. To determine if the effect of 2DG to induce Beclin1 dependent autophagy was limited to PC-3 cells, we also assessed the effect of 2DG in LNCaP cells. As shown in Figure 4, treatment of LNCaP cells

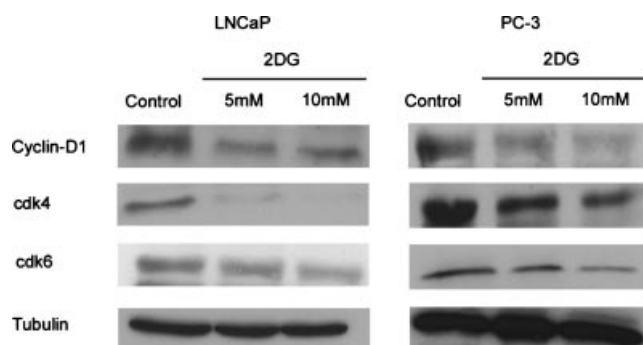


Fig. 2. Effect of 2DG on cell cycle checkpoint proteins. Cyclin-D1, cdk4, and cdk6 were assessed by immunoblot following treatment with 5 and 10 mM 2DG. 2DG decreased expression of Cyclin D1, Cdk4, Cdk6 in LNCaP and PC-3 cells relative to tubulin, which was used to control for protein loading.

with 2DG resulted in similar decreased Beclin1 with siRNA (A) and increased autophagy, as demonstrated by punctate distribution of LC-3 (B,C). As was the case with PC-3 cells, 2DG induced autophagy in LNCaP cells was also dependent on Beclin1 expression.

Effect of 2DG on LC3 and Caspase Activation

To further assess the effect of 2DG on autophagy, we also assessed the expression of LC3 protein by Immunoblot. As shown in Figure 5A, LC3-II protein increased relative to LC3-I protein, as would be expected with induction of autophagy over 72 hr of treatment with 5 or 25 mM 2DG. To begin to determine the effect of Beclin1 on apoptotic proteins such as caspase-3, we assessed the effect of 2DG on the cleaved (activated fragment of caspase-3). As shown in Figure 5B, caspase-3 is cleaved to the active fragment in PC3 cells treated with 2DG. Of note, treatment of Beclin1 siRNA, which was shown to abrogate autophagy (Figs. 3 and 4), allowed increased activation of caspase-3 at lower 2DG concentration, suggesting that Beclin1 and autophagy were associated with resistance to apoptosis with these specific experimental conditions, and at concentrations more relevant to what can be achieved in patients. The effect of autophagy on 2DG induced cytotoxicity was further assessed by a cytotoxicity assay. LNCaP and PC-3 cells were treated with various concentrations of 2DG over 72 hr, with and without beclin1 siRNA or Lamin control, and assessed by cell counts with trypan blue (Fig. 6). Both LNCaP cells and PC-3 cells were inhibited by 2DG in a dose dependent fashion, and cytotoxicity increased with Beclin1 siRNA, consistent with the hypothesis that autophagy was a mechanism of resistance of 2DG induced cytotoxicity.

Beclin-1 Expression in Human Tissue

Because of the dependence of therapeutic starvation-induced autophagy on Beclin1, it would be important to develop this as a translational marker of clinical trials that develop agents that induce metabolic starvation. To determine the feasibility of measuring Beclin1 expression in human prostate tissue, we stained a human prostate tissue microarray by immunohistochemistry (IHC). As shown in Figure 7, the characteristic staining of beclin1 was in epithelial cells in normal (Fig. 7A) and cancer (Fig. 7B,C). As shown in Figure 7D, Beclin1 staining intensity (scored by a single pathologist, M.M., from 0 to 3) was increased in tumor tissue compared to normal tissue. In an effort to develop a standardized methodology for quantifying Beclin1, for use in clinical trial correlates, we performed quantitative digitized image analysis of the tissue microarray. As shown in Figure 8, a color decomposition analysis of tissue staining generated the corresponding measures for integrated staining intensity, effective staining area and effective staining intensity. Thus, Beclin1 can be assessed effectively with IHC in human tissue and quantified by automated digitized imaging, providing a complete assessment methodology to test in clinical studies.

DISCUSSION

Targeting metabolism is an attractive new paradigm for investigation because of increased metabolic fragility of cancer. The understanding and development of clinically available therapies capable of modulating metabolism is critically important. We found that 2DG, a prototypical inhibitor of glycolysis, was cytotoxic in prostate cancer cells. Additionally, we found that 2DG induced the state of autophagy, now known to modulate the effectiveness of targeting metabolism in tumor cells. Autophagy is thought to be a resistance mechanism to cellular stress, or, alternatively, if left to completion, a cause of cell death. Additionally, we demonstrated the importance of Beclin1 as a regulator of autophagy and established methodology to quantitatively assess Beclin1 in human tissue. These data, therefore, support future translational efforts by providing a rationale to assess therapeutics that target metabolism, by demonstrating that autophagy may be a mechanism that modulates cell death, by providing support for the importance of Beclin1 as a regulator of autophagy, and by establishing methodology for the assessment of Beclin1 in patient material in future clinical trials.

The finding that 2DG induced autophagy is important because this may represent either a mechanism of cell death or survival that warrants further study with agents developed for therapeutic starvation such as

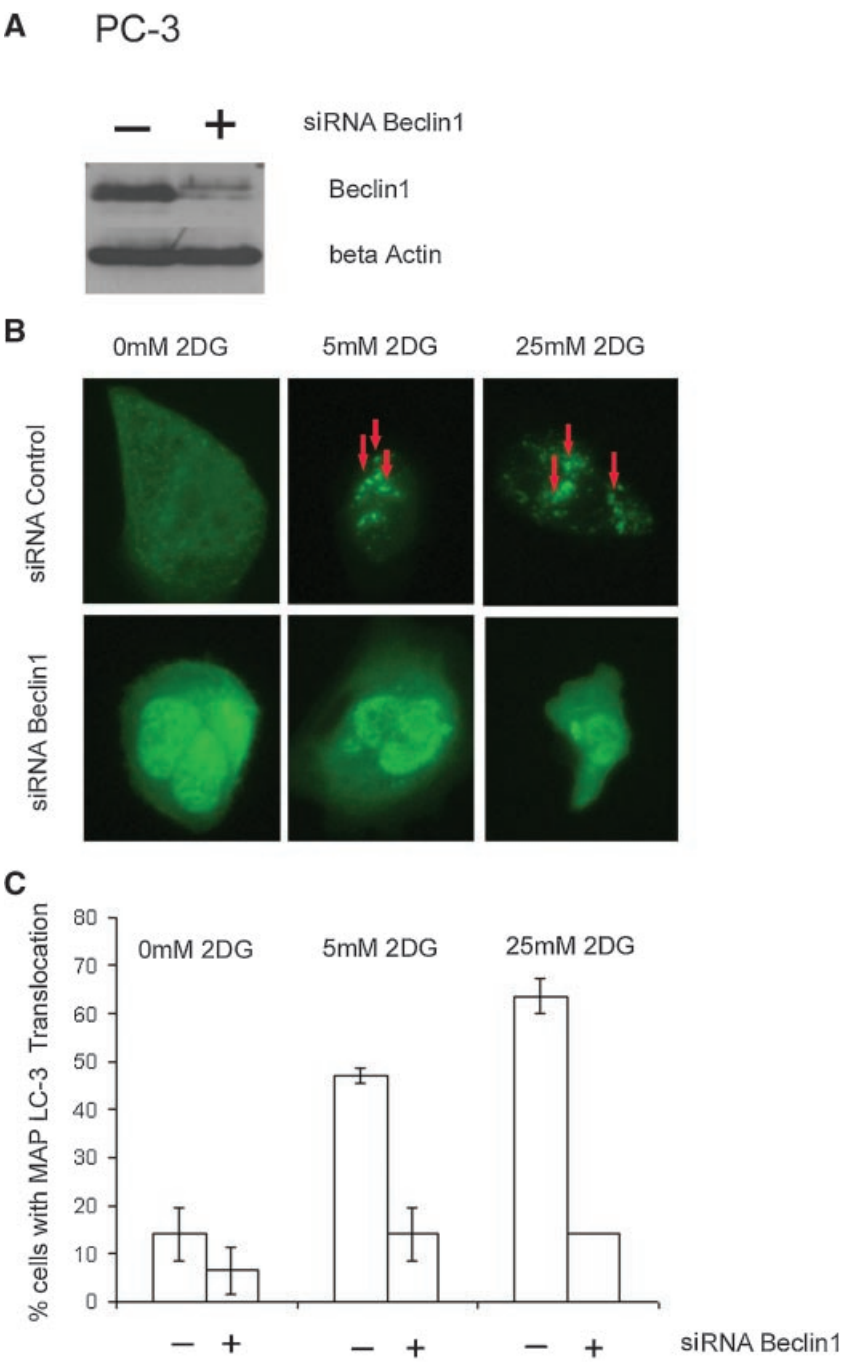


Fig. 3. Induction of Beclin1-dependent autophagy by 2DG in human PC3 cells. **A:** Western blot for Beclin1 and the actin control of PC3 cells treated with Beclin1 siRNA (+) or lamin control (–) siRNA. Beclin1 protein levels were reduced specifically by Beclin1 siRNA. **B:** Representative examples of predominantly diffuse EGFP-LC3 localization without 2DG (0 mM 2DG) and membrane translocation (red arrows) upon 2DG treatment (5 and 25 mM) in the upper row are shown. This punctate pattern represents the localization of the marker GFP-LC3 in autophagosome formation. The localization of GFP-LC3 is abrogated by Beclin1 siRNA (all three lower panels in 3B). **C:** Quantitation of the percentage of counted cells that contained EGFP-LC3 localization indicative of autophagy after treatment with 2DG. A decrease in the percentage of cells with punctate localization of EGFP-LC3 was noted with treatment of Beclin1 siRNA. Each bar represents the percentage of cells with the translocation \pm SEM.

2DG. Autophagy is conserved, genetically controlled catabolic response to starvation whereby cells self-digest intracellular organelles by targeting them for degradation in lysosomes to generate energy. This may

serve to regulate normal turnover of organelles and to remove those with compromised function to maintain homeostasis. Autophagy can also be a survival mechanism during periods of starvation where self-

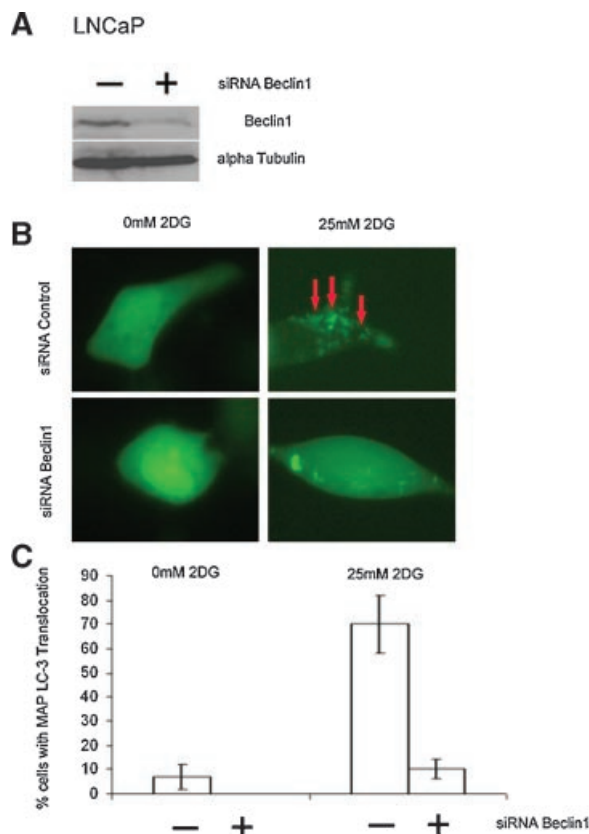


Fig. 4. Induction of Beclin1-dependent autophagy by 2DG in human LNCaP cells. **A:** Western blot for Beclin1 and the actin control of LNCaP cells treated with lamin control siRNA or Beclin1 siRNA. **B:** Representative examples of predominantly diffuse EGFP-LC3 localization without 2DG (0 mM 2-DG) and membrane translocation (red arrows) upon 2DG treatment (25 mM) in the upper row are shown. This punctate pattern represents the localization of the marker GFP-LC3 in autophagosome formation. The localization of GFP-LC3 is abrogated by Beclin1 siRNA (lower panels in 4B). **C:** Quantitation of the EGFP-LC3 localization showing induction of autophagy with 2-DG that is inhibited by siRNA for Beclin1. Each bar represents the percentage of cells with the translocation \pm SEM.

digestion provides an alternative energy source and facilitates the disposal of unfolded proteins under stress conditions. It has recently become clear that normal and tumor cells require the catabolic process of autophagy to survive nutrient deprivation [11]. We found that autophagy was dependent on Beclin1 (Figs. 3 and 4) and functioned as a survival mechanism under our experimental conditions (Figs. 5 and 6), which is consistent with prior studies [16]. We also found that Beclin1 can be detected in human tumor by IHC, establishing the feasibility of measuring Beclin1 in prostate tissue (Figs. 7 and 8), and realize that additional studies would be needed to determine if intensity of expression is associated with the propensity of tumor to undergo autophagy. The implication of measuring

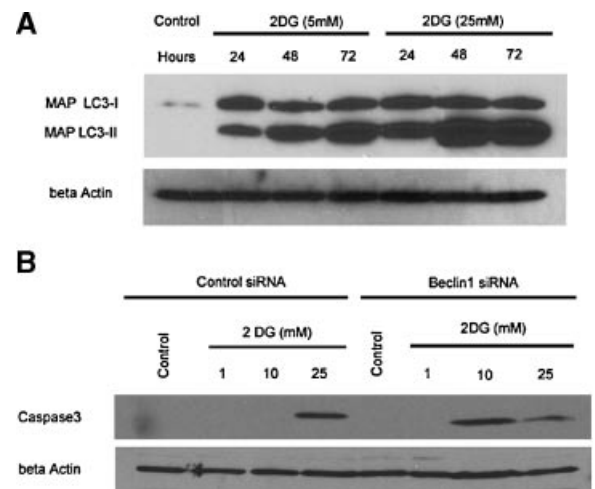


Fig. 5. Effect of 2DG on LC3 protein (**A**) and caspase3 (**B**). LC3-I and LC3-II protein is shown by immunoblot after treatment with 5 and 25 mM of 2DG at 24, 48, and 72 hr showing the characteristic increase in proportion of LC3-II/LC3-I expected with induction of autophagy (**A**). To determine the importance of Beclin1 in 2DG induced apoptosis, we assessed the activated cleavage product of caspase3 in PC3 cells by immunoblot after treatment with 2DG (**B**). Cells were treated with Lamin siRNA (control) or Beclin1 siRNA demonstrating activation and cleavage of caspase3 after treatment with 10 mM 2DG only in the setting of Beclin1 siRNA treatment, which decreased Beclin1 expression (Figs. 3A and 4A) and decreases autophagy (Figs. 3B and 4B). After treatment with 25 mM 2DG, caspase3 is activated and cleaved regardless of beclin1 expression. Actin was used as a control for equal protein loading.

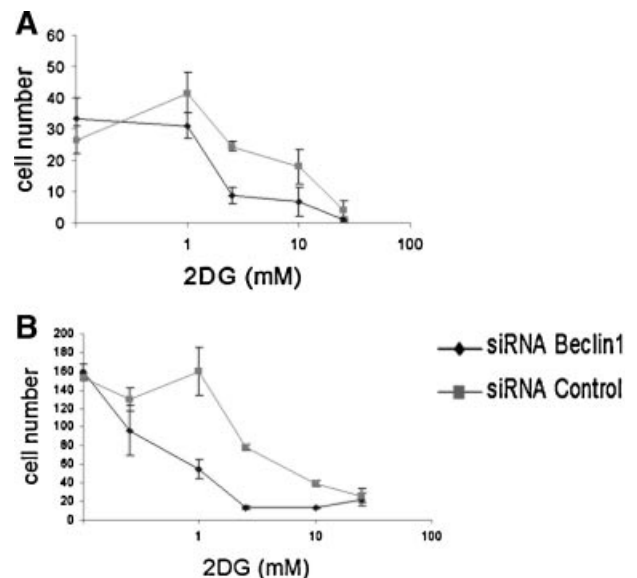


Fig. 6. Effect of autophagy on 2DG induced cytotoxicity. LNCaP (**A**) and PC-3 (**B**) cells were treated with various concentrations of 2DG over 72 hr, with and without beclin1 siRNA or Lamin control, and assessed by cell counts with trypan blue. Both LNCaP cells and PC-3 cells were inhibited by 2DG in a dose dependent fashion, and cytotoxicity increased with Beclin1 siRNA. Experiments were performed in triplicate \pm SEM.

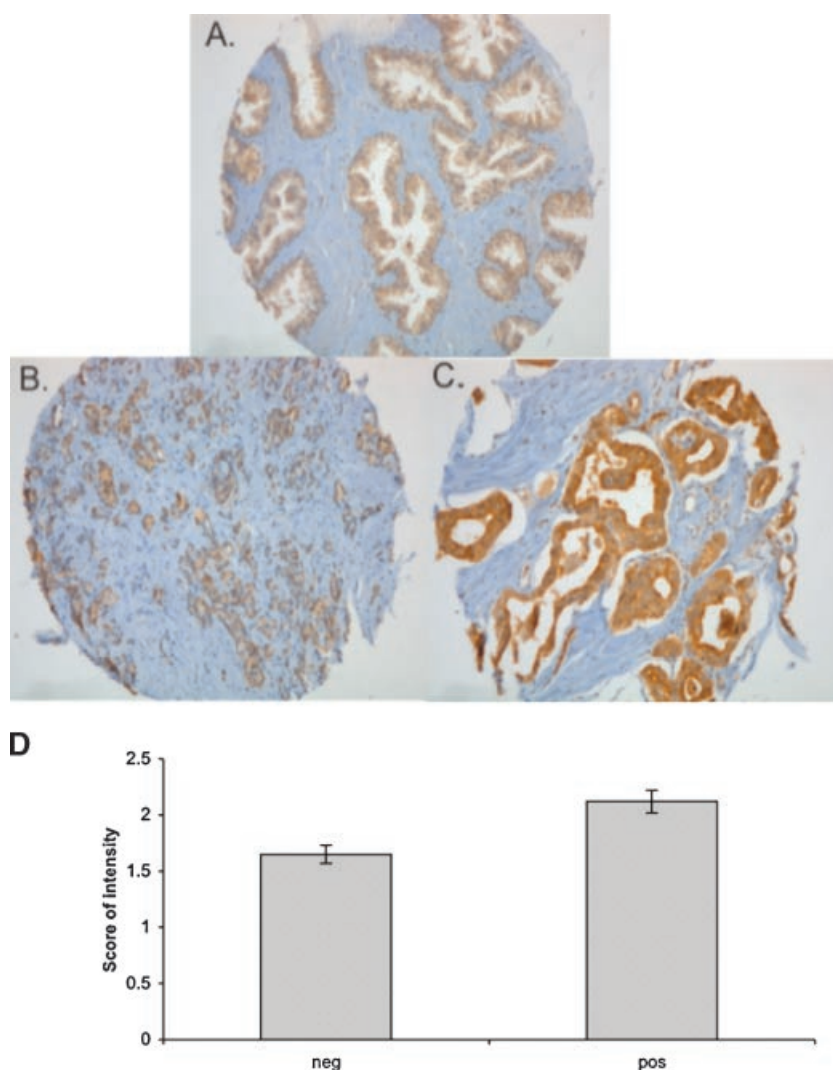


Fig. 7. Tissue microarray of Beclin I staining. IHC of Beclin I from the CINJ prostate TMA. Staining is shown for Beclin I in normal (A) and 1+ (B) and 3+ (C) intensity in tumor. D: Bar graph of percentage of patient tissue microarrays with Beclin I staining in normal tissue (neg) and cancer (pos).

beclin1 in human tumor is currently unclear, and the current assessment was focused to demonstrate the feasibility of assessing beclin1 by IHC in a small group of patient samples, and to quantify the intensity for use in larger clinical studies. This may be particularly important in prostate cancer because multiple studies have demonstrated that the *beclin1* (*atg6*, *vps30*) gene is critical for autophagy to occur and allelic loss occurs with high frequency in prostate cancers [17]. Establishing the role of autophagy in prostate cancer is, therefore, an important step toward understanding the disease process and for the development of new treatments that modulate metabolism [18]. Furthermore, prior studies have demonstrated that activation of the PI-3K/Pten/Akt pathway also promotes glyco-

lysis (in part through up-regulation of glycolytic enzymes and glucose transporters), and stimulates protein synthesis while inhibiting autophagy [4]. Thus, one of the most important events in prostate cancer profoundly alters the cellular metabolic state by increasing energy demand (stimulation of protein synthesis) while promoting inefficient energy production (dependence on glycolysis) and inhibiting catabolism (autophagy).

Therefore, it is our hypothesis that the oncogenic switch from aerobic to glycolytic metabolism, known for decades as the "Warburg effect," causes tumor cells to be predisposed to metabolic catastrophe where constitutive growth signals and inefficient energy production impair their ability to adapt to metabolic

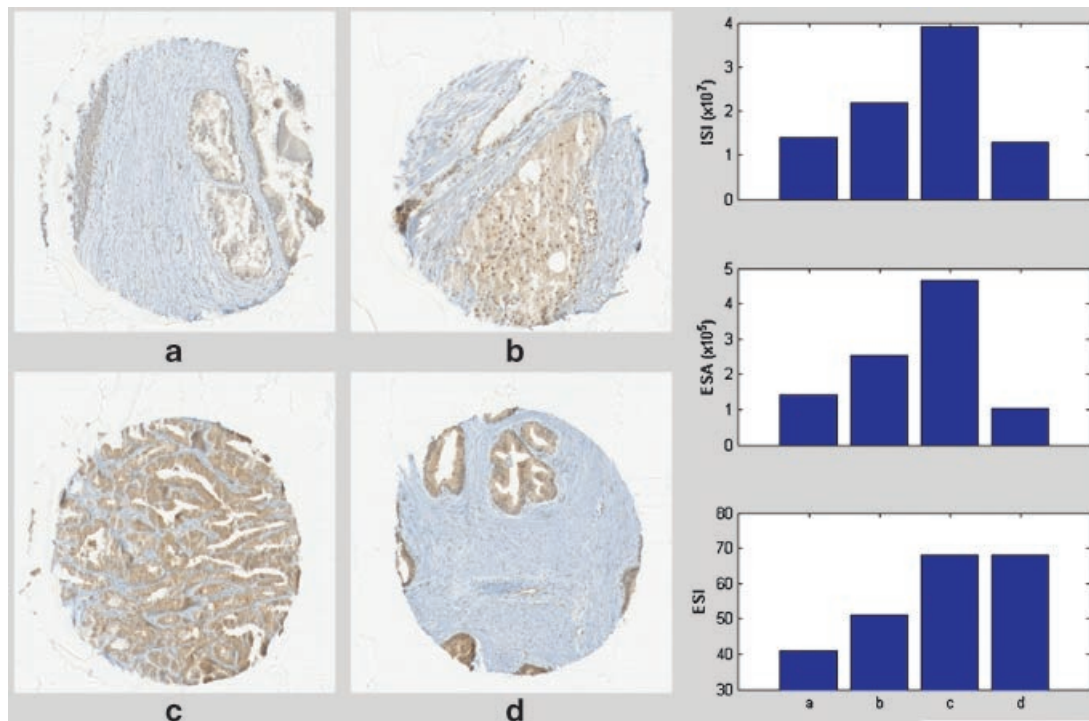


Fig. 8. Quantitative digitized imaging of tissue microarray. As shown in panels a–d, arrays were imaged using a 40 \times volume scan on a Trestle/Clariant whole slide scanner. The diagram located in the right hand column of the figure was generated using color decomposition analysis and shows measures for ISA, ESA, and ESI as previously demonstrated [15].

stress [10]. This fundamental difference between normal and tumor cells has yet to be exploited effectively in the clinic. Our data supports the importance of autophagy in the study of such approaches and gives direction toward the clinical translation of this important paradigm.

CONFLICT OF INTEREST

Views and opinions of, and endorsements by, the authors do not necessarily reflect those of the Department of Defense.

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Warburg science goes to the bedside: A phase I trial of 2-deoxyglucose in patients with prostate cancer and advanced malignancies.

Sub-category:
Prostate Cancer

Category:
Genitourinary Cancer

Meeting:
2008 ASCO Annual Meeting

Abstract No:
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Author(s):
R. S. DiPaola, M. N. Stein, S. Goodin, S. Eddy, E. H. Rubin, S. Doyle-Lindrud, D. Dvorzhinski, S. Beers, W. J. Shih, E. White

Abstract:

Background: A profound, but therapeutically unexploited, difference between cancer and normal tissues is the preferential utilization of glycolysis (the 'Warburg effect') for energy by cancer cells. Additionally, similar to mechanisms of chemotherapy resistance, potential mechanisms of cancer cell resistance to starvation have recently emerged. One pathway by which cells survive periods of metabolic stress is thought to be autophagy, which is a catabolic process of organelle digestion that creates ATP during periods of nutrient limitation and is regulated by the protein Beclin1. **Methods:** We developed this novel paradigm in pre-clinical models and a phase I clinical trial. Preclinically, we used immortalized mouse epithelial prostate cells, as well as PC-3 and LNCaP cell lines, and a transfected pEGFP-LC3 autophagy marker construct to assess cytotoxicity and autophagy induction by 2-deoxyglucose (DG). In the clinic, eligible patients receive DG orally on days 1-14 of a 21 day cycle in cohorts of 3 in a dose escalating manner. Planned correlative assessments in patients included PET scans at baseline and day 2, as a potential marker of DG uptake, Beclin1 in initial tumor blocks, and LC3 protein in peripheral blood mononuclear cells as a potential marker of autophagy. **Results:** In preclinical models, we demonstrated cytotoxicity and induction of autophagy, which was dependent on Beclin1 expression. To establish methods for the clinical trial, we stained a human prostate TMA (>35 patients) for Beclin1 by IHC. In the clinical study, 6 patients have been treated at doses 30 and 45 mg/kg/day orally and a 3rd cohort is accruing currently at 60 mg/kg. Therapy was well tolerated with no dose-limiting toxicity. Of three patients with prostate cancer, one patient has received more than 11 cycles with a stable PSA for over 6 cycles. Of three patients in which PET was performed at baseline and follow-up, one patient had marked decrease in tumor site SUV and a second patient a minor decrease. Accrual is ongoing. Additional PET and assessment of LC3 and Beclin1 correlatives are ongoing. **Conclusions:** These initial data support the safety of DG and translational advancement of the rapidly developing paradigm of targeting the metabolic fragility of cancer.

Abstract Disclosures

Abstracts that were granted an exception in accordance with ASCO's Conflict of Interest Policy and are designated with a caret symbol (^) here and in the print version.

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